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Effect of Maternal Smoking on Plasma and Urinary Measures of Vitamin E Isoforms in the First Month after Extreme Preterm Birth

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Abstract

We examined the effect of maternal smoking on plasma and urinary levels of vitamin E isoforms in premature infants. Maternal smoking during pregnancy decreased infant plasma alpha- and gamma-tocopherol concentrations at 1 and 4 weeks, with 45% of infants of smokers deficient in alpha-tocopherol at one month after birth.

Keywords

vitamin E; preterm; alpha-tocopherol; gamma-tocopherol; smoking; pregnancy; prematurity

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Vitamin E consists of eight different antioxidant isoforms with potentially differing contributions to diseases whose pathobiology centers on oxidative stress or damage. Two of these isoforms predominate in the human diet and body. The most abundant tissue vitamin E isoform, alpha-tocopherol, has been hypothesized to have a beneficial role in dampening the inflammatory cascade in multiple conditions associated with prematurity, such as bronchopulmonary dysplasia(1–4), retinopathy of prematurity(5–9), and intraventricular hemorrhage(10, 11). The next most common isoform, gamma-tocopherol, is not as well studied in neonates. Although it is known to be a potent antioxidant, it appears to be pro-inflammatory in some disease states, and modifies the protective benefit of alpha-tocopherol(12–14). Alpha- carboxyethyl hydroxychroman (CEHC) and gamma-CEHC are the main urinary metabolites of alpha- and gamma-tocopherol, respectively(15–18). Maternal smoking has been demonstrated to affect the amount of tocopherols present in umbilical cord blood samples, and is postulated to affect the infant's ability to handle oxidative stress(19). Measurement of these molecules in neonates has previously necessitated a blood draw for plasma or whole blood, which poses technical and medical challenges in small preterm infants. The objectives of this study are to determine the effects of maternal smoking on measurements of tocopherols and their urinary metabolites in preterm infants in the first postnatal month. We also sought to determine whether urinary and plasma levels of alphaand gamma-tocopherol and their respective metabolites correlate with one another in the first weeks after birth. This is important for both research and clinical purposes, as it would reduce the need for a blood draw to measure these molecules, and allow for ease of multiple, longitudinal assessments of tocopherol metabolites in research studies.

Methods

We studied 41 premature infants in whom we had available blood and urine samples at either of 2 different time points (1 week after birth and 1 month after birth), to evaluate correlations between plasma alpha- and gamma-tocopherol and their primary urinary metabolites, alpha- and gamma-CEHC. In addition, we analyzed urinary concentrations of F2-isoprostanes, a biomarker of oxidative stress. We had available blood samples at both time points for 30 premature infants, and urine samples at both time points for 32 infants.

Paired blood and urine samples from the same day were obtained from every infant, though not all infants were able to provide samples at both time points. The first collection occurred at a median of postnatal day 6 and the second collection occurred at a median of postnatal day 27.

Blood and urine samples from 2 time points were obtained from premature infants born at <29 weeks gestational age enrolled in the Improving Prematurity-Related Respiratory Outcomes at Vanderbilt (IMPROV) cohort (U01 HL101456), part of the multicenter Prematurity and Respiratory Outcomes Program (PROP; U01 HL101794), a prospective, observational birth cohort of preterm infants <29 weeks of gestation at birth. The infants in this study were enrolled at Vanderbilt between September 2011 and December 2013. Specimens for biomarker analysis were collected in the first month after birth with the goal of identifying mechanisms that predict pulmonary outcomes, such as bronchopulmonary

dysplasia (BPD) and respiratory morbidity during the first year after birth. The methods and descriptions of the PROP study have been published (20–23) and are available at clinicaltrials.gov. (<https://clinicaltrials.gov/ct2/show/NCT01460576>). Maternal smoking during pregnancy was assessed via standardized questionnaires and chart review, limited to “yes/no” data, without further quantification of the amount of smoking exposure.

Quantification of tocopherol isoforms was performed using gas chromatography and mass spectrometry. Detailed descriptions of the methods used can be found in the Appendix (available at www.jpeds.com).

Statistical Analyses

Continuous variables were summarized using the median, 25th, and 75th percentile with interquartile range [IQR], and categorical variables were summarized using percentages. Spearman rank correlation was used to estimate bivariate associations between all pairwise combinations of alpha- and gamma- tocopherol measured in the urine and the plasma. The Pearson test was used to test differences among categorical variables. The Wilcoxon signed rank test for paired data was used to test if tocopherol levels changed significantly from the first to the second collection time. For comparisons across independent groups (eg, smokers versus non-smokers), we used the Wilcoxon rank sum test (2 groups) or the Kruskal-Wallis test (more than 2 groups). Rank methods were used to mitigate the impact of outliers and alleviate the need to consider transformations. As we report the results of all associations tested, we did not make any formal corrections for multiple comparisons.

Results

The overall maternal and infant characteristics of our cohort with stratification by maternal smoking during pregnancy is shown in Table I. There are no significant differences among the characteristics studied. We further stratified the same maternal and infant characteristics by maternal race with no significant differences noted between the groups. (Table 2; available at www.jpeds.com).

Between 1 week and 1 month after birth, the median plasma alpha-tocopherol concentration decreased in our premature subjects from a median [IQR] of 22 $\mu\text{mol/L}$ [17, 28] to 19 $\mu\text{mol/L}$ [14, 26] ($n=30$ paired samples, Wilcoxon signed rank test, P value .02) and median plasma gamma tocopherol decreased from 6.4 $\mu\text{mol/L}$ [5.3, 10.1] to 2.7 $\mu\text{mol/L}$ [1.9, 5.0] (Wilcoxon signed rank p -value <0.001) (Figure 2; available at www.jpeds.com). There were nonsignificant increases in urinary CEHC isoforms over the same time period. (Table 1.

At each of the 2 time points, we observed significantly lower plasma alphetocopherol in premature infants of mothers who smoked ($n=15$) during pregnancy compared with infants of nonsmokers ($n=26$) (Table 1). At one week after birth, the median [IQR] of alpha-tocopherol was 27 $\mu\text{mol/L}$ [21, 33] in infants of nonsmokers versus 19 $\mu\text{mol/L}$ [16, 20] in infants of smokers, ($p=0.007$). At one month of age, median alpha-tocopherol was 22 $\mu\text{mol/L}$ [15.5, 28] in infants of nonsmokers versus 14 $\mu\text{mol/L}$ [7, 17] in infants of smokers, ($p=0.002$) (Figure 3; available at www.jpeds.com). For gamma- tocopherol, the median value at one month was 4.1 $\mu\text{mol/L}$ [2.7, 8.1] in the infants of nonsmokers versus 2.0 $\mu\text{mol/L}$

[1.7, 2.7] in the infants of smokers, ($p=0.02$). Although we observed reductions in gamma-tocopherol at 1 week, and in alpha-CEHC and gamma-CEHC at both time points, when comparing infants of smokers with infants of nonsmokers, they were not statistically significant. We did not detect any difference in production of urinary F2-isoprostanes, a marker of oxidative stress, between infants of smokers versus nonsmokers. (Table 1). Between one week and one month after birth, we did not observe any differences in the rate of decrease in plasma-tocopherol isoforms between infants of smokers and nonsmokers ($p=0.40$ for alpha-tocopherol and $p=0.34$ for gamma tocopherol) (Figure 4; available at www.jpeds.com).

Though cutoffs for alpha tocopherol deficiency are not well defined for preterm neonates, use of the current cutoff for deficiency of alpha-tocopherol at $12.0 \mu\text{mol/L}$ (24) shows that 42% of the premature infants of smokers were deficient at 1 month after birth, compared with 5% of premature infants born to nonsmokers ($p=0.008$).

We next looked at the effect of TPN on the same biomarkers at one month after birth, when 44% of the infants remained on TPN and the other 56% were on full enteral feeds. We noted significant increases in measured plasma alpha-tocopherol ($p=0.01$) and gamma-tocopherol concentrations among those infants still on TPN ($p<0.001$). (Table 3; available at www.jpeds.com)

We then stratified the relationship between tocopherol isoforms and maternal smoking by whether or not the infant remained on TPN at one month after birth (all infants were still on TPN at one week) (Figure 1 and Table 4 [available at www.jpeds.com]). We found consistent decreases in the concentrations of alpha- and gamma-tocopherol for maternal smoking and increases in concentrations if TPN was present, such that infants of smokers who were no longer on TPN at one month had the lowest concentrations of both alpha-tocopherol ($p<0.001$) and gamma-tocopherol ($p<0.001$) and were most likely to be deficient ($p=0.037$). (Table 4) Urinary F2-isoprostanes, when stratified by the presence or absence of TPN, were nonsignificantly increased in infants on TPN ($p=0.12$). (Table 3)

We were unable to detect significant correlations between plasma alpha- or gamma-tocopherol concentrations and their paired urinary metabolites alpha-CEHC and gamma-CEHC at either of the 2 time points. At 1 week after birth, both plasma tocopherols and their corresponding urinary metabolites were negatively correlated, and at 1 month after birth, they were positively correlated, but this was not significant.

Measurements of the plasma isoforms were positively and significantly correlated over time: plasma alpha tocopherol at one week and one month, (Spearman correlation coefficient, $CC=0.61$, $p=0.003$) and plasma gamma tocopherol at one week and one month, ($CC=0.46$, $p=0.016$). Meanwhile, measurements of the urinary isoforms were not correlated over time; neither urinary alpha-CEHC at 1 week and 1 month ($CC=-0.26$, $p=0.11$) nor urinary gamma-CEHC at one week and one month ($CC=0.01$, $p=0.34$). We were not able to effectively stratify this analysis by TPN status due to small sample size, given that all subjects were on TPN at one week.

Discussion

In extremely preterm infants, plasma alpha- and gamma-tocopherol decline over the first month after birth regardless of maternal smoking status during pregnancy. Infants of smoking mothers who are no longer on TPN at one month after birth are at the highest risk of alpha-tocopherol deficiency. The contribution of declining levels of plasma tocopherol to oxidant stress and specific health outcomes in preterm infants is not known but is an area worthy of study.

In premature infants, maternal smoking during pregnancy significantly decreases plasma alpha tocopherol, leading to higher rates of deficiency by one month after birth. Remaining on TPN at one month after birth significantly increases both plasma alpha-tocopherol concentrations, and gamma-tocopherol concentrations. Depending on the oil source used for lipid emulsions and vitamin E in TPN, the vitamin E isoform composition may vary. For example, corn and soybean oils contain predominantly gamma-tocopherol, whereas sunflower and safflower oils contain predominantly alpha-tocopherol.(12) For the infants in this study the source of lipid emulsion was Intrapilid 20% consisting of 20% soybean oil. Gamma-tocopherol is known to have a different biological profile from alphetocopherol in terms of downstream oxidative and inflammatory effects,(12) and its independent associations and interactions with alpha-tocopherol on diseases of prematurity are unknown. Cook-Mills et al have reported that in term neonates, increasing gamma-tocopherol concentrations were associated with reductions in FEV1 ($p=0.01$) and FVC ($p=0.01$), whereas increasing alpha-tocopherol was associated with increases in FVC ($p=0.01$) (14).

These observations in the plasma and urine of extremely premature infants are consistent with reports that smoking decreases alpha tocopherol concentrations in both pregnant mothers and their term offspring(19, 25). The role of alpha tocopherol in lung development and antioxidant defense is well known. The association between maternal smoking during pregnancy and reduced alpha tocopherol in their preterm infants points to a potentially modifiable mechanism underlying the link between maternal smoking and poor pulmonary outcomes, including bronchopulmonary dysplasia and late respiratory disease in preterm infants (26), as has been demonstrated with vitamin C (27). This information will be helpful in future trials of prenatal or postnatal tocopherol supplementation, as infants of smokers might be a targeted risk group for study.

CEHC metabolites of tocopherol are easily transferred across the placenta, whereas alpha and gamma tocopherol are not as easily transferred, though they do equilibrate during pregnancy (28, 29). Changes in infant metabolism of tocopherols over this time period may also be present, given the switch in the directionality we observed in the correlations between plasma isoforms and their urinary metabolites, and future studies at one month of age may be more fruitful.

Our study was underpowered to determine whether plasma tocopherol isoforms are correlated with their urinary metabolites and urinary F2-isoprostanes in premature neonates. Larger, prospective studies are needed to determine whether measuring urinary metabolites reduces the need for blood draws in ascertaining the tocopherol status of premature infants

and whether urinary or plasma tocopherol levels are associated with their long-term respiratory outcomes.

Strengths of our study were the availability of blood and urine biospecimens and a robust clinical dataset from preterm infants across time in the early postnatal period. Because all infants were treated at a single center, TPN formulation and source were the same, as were other protocols used in treating the infants.

Limitations included a small sample size and a single-center study design.⁽³⁰⁾ We were not able to assess other nutritional factors, such as vitamin C or fish oil derived fatty acids, which may be relevant confounders for an oxidative effect of smoking on vitamin E. Our questionnaire data did not quantify the amount of maternal smoking exposure during pregnancy, which would be important in assessing for dose-response on infant vitamin E isoforms. Replication of our findings in a larger cohort of preterm infants will be important for future research.

Conclusions

In summary, maternal smoking decreases early life plasma concentrations of alpha-tocopherol in their premature infants and leads to increased likelihood of tocopherol deficiency by one month of age. Infants of smokers who are no longer on TPN are at the greatest risk of deficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations and Acronyms

nmol	nanomole
μmol	micromole
L	Liter
mg Cr	milligrams Creatinine
CEHC	carboxyethyl hydroxychroman
Alpha-CEHC	3-(6-hydroxy-2,5,7,8-tetramethyl-3,4-dihydro-2H-1-benzopyran-2-yl)propanoic acid
Gamma-CEHC	3-(2,7,8-trimethyl-3,4-dihydro-2H-1-benzopyran-2-yl)propanoic acid

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Figure 1.

Effects of maternal smoking and TPN on infant plasma tocopherol isoforms one month after birth. Maternal smoking was noted to decrease measured concentrations of both isoforms, while TPN was noted to increase them. Individual data points are jittered (randomly moved up or down) along the horizontal axis so as not to overlap visually. The boxplots are (usual) Tukey boxplots. The boxes contains that 25th and 75th percentiles, the line in the middle is the median. The whiskers (lines) extend out to lesser of 1) the most extreme value or 2) $1.5 \times \text{IQR}$ (interquartile range). Comparison across all groups by isoform was done via Kruskal-Wallis test, (p-value alpha-tocopherol=0.002, p-value gamma-tocopherol =0.001.)

Alpha-tocopherol deficiency, defined as a concentration less than 12.0 $\mu\text{mol/L}$, was present in 50% of infants in the mother smoker, infant off TPN group; 25% of the infants in the mother smoker, infant on TPN group; 10% of the mother nonsmoker, infant off TPN group; and 0% in the mother nonsmoker, infant on TPN group. Pearson test (p-value=0.037).

Figure 2.

Plasma alpha and gamma tocopherol over time in preterm neonates. Heavy dark line signifies the median values, individual dots represent an individual patient, with before and after measurements connected by lines. Significant decreases in both isoforms were observed, median [IQR] of 22 μmol/L [17, 28] to 19 μmol/L [14, 26] (n=30 paired samples, Wilcoxon signed rank p-value 0.02) and median plasma gamma tocopherol decreased from 6.4 μmol/L [5.3, 10.1] to 2.7 μmol/L [1.9, 5.0] (n=32 paired samples, Wilcoxon signed rank p-value <0.001).

Figure 3.

Significant decreases in plasma alpha tocopherol were observed in infants of smokers at both time points. At one week after birth, the median [IQR] of alpha-tocopherol was 27 μmol/L [21, 33] in infants of nonsmokers versus 19 μmol/L [16, 20] in infants of smokers, (Wilcoxon rank sum, $p=0.007$). At one month after birth, median alpha-tocopherol was 22 μmol/L [15.5, 28] in infants of nonsmokers versus 14 μmol/L [7, 17] in infants of smokers, (Wilcoxon rank sum, $p=0.002$).

Figure 4.

Decreases in alpha and gamma tocopherol were observed in premature neonates over time, with baseline decreases in both plasma alpha tocopherol and gamma tocopherol in infants of smokers. Rate of decline in alpha- and gamma-tocopherol, however, was not influenced by maternal smoking during pregnancy. (Wilcoxon rank sum, alpha-tocopherol p-value=0.40 and gamma-tocopherol p-value= 0.34)

Table 1

Clinical and Laboratory Characteristics of Maternal/Infant Pairs

	Overall population: N=41 Median [IQR] or n (%)	Nonsmoking mother during pregnancy: n= 26 Median [IQR] or n (%)	Smoking mother during pregnancy: n=15 Median [IQR] or n (%)	P-value: (comparing smoking vs. nonsmoking)
Maternal Characteristics				
Maternal Age (years)	25 [22, 29]	25 [22, 29]	24 [21, 30]	0.58
Maternal Race/Ethnicity				
- Black	11 (27%)	8 (31%)	3 (20%)	0.45 *
- White	30 (73%)	18 (69%)	12 (80%)	
- Hispanic	0 (0%)	0 (0%)	0 (0%)	
Maternal Perinatal Health Factors				
- Diabetes	2 (5%)	0 (0%)	2 (13%)	0.06 *
- Hypertension	11 (27%)	7 (27%)	4 (27%)	0.99 *
- Asthma	1 (2%)	0 (0%)	1 (7%)	0.18 *
- Smoking during pregnancy	15 (37%)	0 (0%)	15 (100%)	
Infant Characteristics				
Infant Gestational Age (weeks)	26 [25, 26]	26 [25, 26]	26 [25, 27]	0.58
Infant Birthweight (grams)	790 [680, 930]	786 [698, 881]	790 [614, 930]	0.76
Infant Sex (Female)	11 (27%)	7 (27%)	4 (27%)	0.99 *
Infant on TPN at one week of life	41 (100%)	26 (100%)	15 (100%)	1
Infant on TPN at one month of life	23 (56%)	13 (50%)	10 (67%)	0.3
Sample collection, 1 st time point (day of life)	6 [5, 8]	6 [5, 7]	6 [5, 8]	0.89
Sample collection, 2 nd time point (day of life)	27 [26, 28]	27 [26, 28]	26 [26, 28]	0.53
Plasma Tocopherol Measurements				
Plasma alpha-tocopherol at one week of life (μmol/L)	22 [17, 28]	27 [21, 33]	19 [16, 20]	0.007
Plasma alpha-tocopherol at one month of life (μmol/L)	19 [14, 26]	22 [16, 28]	14 [7, 17]	0.002
• p-value (comparing one week to one month of life)	0.02			
Plasma gamma-tocopherol at one week of life (μmol/L)	6.4 [5.3, 10.1]	8.6 [5.9, 10.6]	5.7 [5.3, 7.2]	0.095
Plasma gamma-tocopherol at one month of life (μmol/L)	2.7 [1.9, 5.0]	4.1 [2.7, 8.1]	2.0 [1.7, 2.7]	0.02
• p-value (comparing one week to one month of life)	<0.001			
Urinary CEHC and Isoprostane Measurements				
Urinary alpha-CEHC at one week of life (nmol/L)	3.9 [1.9, 9.1]	2.6 [2.1, 7.2]	3.7 [1.8, 19.0]	0.70
Urinary alpha-CEHC at one month of life (nmol/L)	11.3 [2.8, 19.0]	10.4 [2.2, 17.3]	6.3 [0.1, 13.3]	0.64
• p-value (comparing one week to one month of life)	0.26			
Urinary gamma-CEHC at one week of life (nmol/L)	5.9 [2.6, 13.1]	5.9 [4.0, 12.9]	5.7 [2.1, 10.8]	0.80

	Overall population: N=41 Median [IQR] or n (%)	Nonsmoking mother during pregnancy: n= 26 Median [IQR] or n (%)	Smoking mother during pregnancy: n=15 Median [IQR] or n (%)	P-value: (comparing smoking vs. nonsmoking)
Urinary gamma-CEHC at one month of life (nmol/L)	8.2 [3.0, 20.8]	9.4 [3.5, 14.3]	7.5 [0.7, 20.5]	0.55
• p-value (comparing one week to one month of life)	0.52			
Urinary F2-Isoprostanes at one week of life (ng/mg Cr)	4.1 [3.1, 10.0]	4.2 [2.7, 6.9]	4.1 [3.8, 16.3]	0.16
Urinary F2-Isoprostanes at one month of life (ng/mg Cr)	5.6 [3.9, 11.2]	6.1 [3.7, 10.3]	5.6 [4.4, 11.5]	0.79

* denotes analysis conducted with Pearson test. Comparisons of one week to one month use the paired Wilcoxon rank sum. All other results use the Wilcoxon signed rank test.

Table 2

Characteristics of Maternal/Infant Pairs Stratified by Race

	Overall population: N=41 Median [IQR] or n (%)	Black: n= 11 Median [IQR] or n (%)	White: n=30 Median [IQR] or n (%)	P-value: (comparing black vs. white)
Maternal Characteristics				
Maternal Age (years)	25 [22, 29]	28 [21, 31]	25 [22, 29]	0.37
Maternal Perinatal Health Factors				
- Diabetes	2 (5%)	0 (0%)	2 (7%)	0.38 [*]
- Hypertension	11 (27%)	3 (27%)	8 (27%)	0.97 [*]
- Asthma	1 (2%)	0 (0%)	1 (3%)	0.54 [*]
- Smoking during pregnancy	15 (37%)	3 (27%)	12 (40%)	0.45 [*]
Infant Characteristics				
Infant Gestational Age (weeks)	26 [25, 26]	26 [25, 26]	26 [25, 27]	0.54
Infant Birthweight (grams)	790 [680, 930]	750 [634, 885]	790 [708, 919]	0.28
Infant Sex (Female)	11 (27%)	3 (27%)	8 (27%)	0.97 [*]

^{*} denotes analysis conducted with Pearson test, all other statistical comparisons performed using Wilcoxon test.

Table 3

Stratification of Tocopherol Isoforms, CEHC Isoforms, Urinary F2-Isoprostanes at One Month by Infant TPN status

	On TPN (n=18) Median [IQR] or n (%)	Off TPN (n=23) Median [IQR] or n (%)	p-value:
Alpha-tocopherol (μmol/L)	25.1 [15.5, 31.0]	16.0 [8.5, 20.8]	0.01
n(%) of patients with alpha-tocopherol less than 12 μmol/L	1 (7%)	5 (28%)	0.12 *
Gamma-tocopherol (μmol/L)	5.0 [4.1, 8.7]	2.0 [1.6, 2.7]	<0.001
Alpha-CEHC (nmol/L)	14.9 [8.4, 26.2]	3.8 [1.7, 10.2]	0.063
Gamma-CEHC (nmol/L)	10.3 [2.9, 14.3]	7.5 [2.4, 21.7]	0.85
Urinary F2-Isoprostanes(ng/mg Cr)	9.2 [5.6, 13.8]	5.0 [3.7, 8.8]	0.12
Day of Life Sample Collected	28 [26, 28]	27 [26, 28]	0.98
Maternal Smoking during pregnancy	5 (28%)	10 (43%)	0.3 *

* denotes analysis conducted with Pearson test, all other statistical comparisons performed using Wilcoxon test.

Table 4

Stratification of Tocopherol Isoforms, CEHC Isoforms, Urinary F2 Isoprostanes at One Month by Maternal Smoking and Infant TPN status

	Mother smoker, infant off TPN (n=10) Median [IQR]	Mother smoker, infant on PN (n=5) Median [IQR]	Mother nonsmoker, infant off TPN (n=13) Median [IQR]	Mother nonsmoker, infant on TPN (n=13) Median [IQR]	p-value:
Alpha-tocopherol (μmol/L)	11.1 [5.6, 16.8]	14.9 [13.2, 17.6]	19.7 [13.7, 22.1]	28.1 [21.0, 32.9]	0.002
n(%) of patients with alpha-tocopherol less than 12 μmol/L	4 (50%)	1 (25%)	1 (10%)	0 (0%)	0.037*
Gamma-tocopherol (μmol/L)	1.9 [1.5, 2.0]	4.0 [2.9, 5.0]	2.7 [1.9, 3.5]	8.1 [4.6, 10.1]	0.001
Alpha-CEHC (nmol/L)	5.2 [0.1, 7.0]	20.5 [10.0, 29.5]	3.1 [2.0, 11.7]	14.8 [8.3, 19.4]	0.33
Gamma-CEHC (nmol/L)	5.3 [0.7, 20.5]	13.4 [7.6, 17.7]	10.8 [5.3, 21.4]	9.3 [2.9, 12.3]	0.86
Urinary F2-Isoprostanes(ng/mg Cr)	5.6 [4.3, 9.6]	11.5 [5.6, 11.5]	4.4 [3.6, 8.8]	9.1 [5.6, 14.6]	0.43

* denotes analysis conducted with Pearson test, all other statistical comparisons performed using Wilcoxon test.